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ATR-FTIR study of the protonation states of the Glu residue in the multicopper oxidases, CueO and bilirubin oxidase

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ABSTRACT

Redox-induced protonation state changes of the Glu residue in the multicopper oxidases, CueO and bilirubin oxidase (BO), were studied by attenuated total reflectance-Fourier transform infrared spectroscopy. By monitoring IR bands of the carboxylic acid C=O stretch in the wild-type and Glu-to-Gln mutant enzymes the Glu506 of CueO (Glu463 of BO) was found to be unprotonated in the oxidised and protonated in the reduced forms. The results provided direct evidence for proton uptake by the Glu, suggesting it plays a key role in the proton donation to the activated oxygen species in the catalytic cycle.

1. Introduction

CueO is a multicopper oxidase that is localised in a peliplasmic space and is involved in the copper efflux system of *Escherichia coli* [1–3]. The enzyme (57 kDa) binds four copper atoms to form the catalytic centre, which catalyses oxidation of exogenous cuprous (Cu^{I}) ions to cupric (Cu^{II}) ions coupled with O_2 reduction:

$$4Cu^{I} + 4H^{+} + O_2 \rightarrow 4Cu^{II} + 2H_2O$$

First, an electron from the cuprous ion is transferred to a mononuclear type I (T1) Cu site in CueO and then to a trinuclear site that is composed of one type II and two type III (T2/T3) Cu atoms (Fig. 1). The binding and reduction of O_2 occur in the T2/T3 site, however the detailed reaction mechanism is yet unclear. In the X-ray crystal structure of CueO, the Glu506 is the closest carboxylic group to one of the T3 Cu ions among the acidic amino acid residues (4.9 Å according to PDB entry 1KV7 [2]). One of the oxygen atoms of the carboxy group of Glu506 is within hydrogen bonding distance of the N_π of His499 (3.0 Å) and the oxygen (2.9 Å) of the water molecule that is hydrogen-bonded (2.7 Å) to the hydroxo bridge of the two T3 Cu ions. The other oxygen atom is within hydrogen bonding distance of the N_π of His143 (3.4 Å) and the oxygen of a water molecule (3.4 Å) for access to the external media (Fig. 1).

The acidic residue that is equivalent to Glu506 is genetically and/or structurally conserved among the multicopper oxidase family, such as Glu463 in the bilirubin oxidase (BO) from *Myrothecium verrucaria* [4], Glu498 in the endospore coat protein CotA from *Bacuillus subtilis* (1GSK.pdb) [5], Glu510 of ascorbate oxidase (1AOZ.pdb) [6], Glu487 of yeast (*Saccharomyces cerevisiae*) Fet3p (1ZPU.pdb) [7] and Asp456 of the fungus *Trametes versicolor* laccase (1KYA.pdb) [8]. It seems that the acidic residue located in the position is functionally essential for O_2 reduction in multicopper oxidases. As an exceptional example, it is noted that in the fungus *Melanocarpus albomyces* laccase, the equivalent acidic residue is missing but some water molecules occupy the space (2Q9O.pdb) [9].

Previously, Kataoka et al. [10] examined the function of Glu506 in CueO using a single-mutant Glu506Gln and a double-mutant Cys500Ser/Glu506Gln, of which the latter lacks the T1 Cu. The Glu506Gln enzyme contained four Cu atoms per protein molecule

Abbreviations: ATR-FTIR, attenuated total reflectance-Fourier transform infrared; BO, bilirubin oxidase

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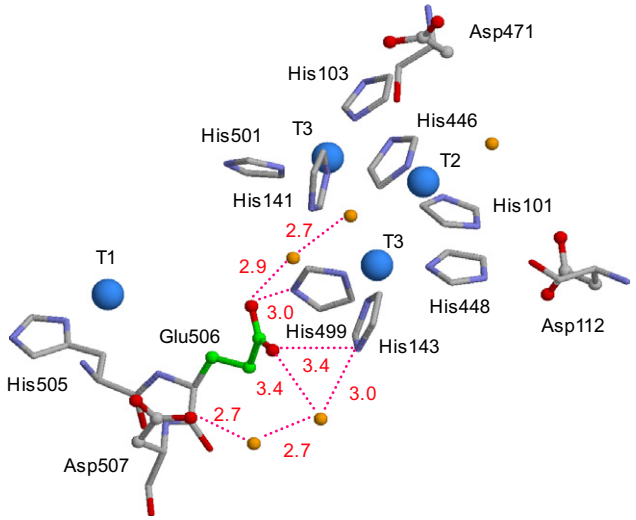

Fig. 1. Molecular structure of the catalytic site of CueO. Type I, Type II and Type III copper atoms (spheres in blue) are labelled as T1, T2 and T3, respectively. The carbon atoms of the Glu506 headgroup are emphasised in green. Oxygen atoms of water molecules (or hydroxy ions) are shown as spheres in yellow. Possible hydrogen bonds mentioned in the text are indicated by dotted lines with the distances in Å. The structure was drawn using PDB entry 1KV7 [2].

and its oxidised (resting) form showed absorption, circular dichroism and electron paramagnetic resonance spectra similar to those of the wild-type (WT) enzyme. Its catalytic activity, however, was extremely low. Injection of O_2 to the four-electron reduced form of Glu506Gln generated an intermediate containing a stable activated oxygen species. In the intermediate state the T1 Cu was re-oxidised, which was confirmed by the recovery of absorption band at 610 nm. The intermediate was also characterised by absorption maxima at 315 and 350 nm and an EPR signal with *g* values of 2.12 and 1.94, indicating that the O–O bond was cleaved and three T2/T3 Cu atoms were re-oxidised and magnetically coupled [10]. The T1 depleted Glu506Gln enzyme (Cys500Ser/Glu506Gln) "as prepared" was in a stable intermediate-like form rather than the conventional oxidised form. These results suggested that Glu506 could be a proton donor to the activated oxygen species during O_2 reduction. In the case of Fet3p, the kinetic analysis using T1 Cu depleted Glu487 mutants suggested that Glu487 donated a proton to the peroxide intermediate [11]. For CotA, X-ray crystallography and spectroscopic analysis of the Glu498 mutant enzymes suggested that Glu498 functioned in the protonation events as well as provided structural stabilization [12]. In principle, all of the papers above agreed that the acidic residue in the proper position plays a key role in proton translocation during O_2 reduction.

In this paper, we studied redox-induced protonation state changes in the acidic residues of the WT and Glu506Gln mutant enzymes of CueO by using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy combined with electrochemistry. Similar experiments were performed in the WT and Glu463Gln mutant enzymes of BO from *M. verrucaria* to confirm the function of the acidic residue. Recent X-ray crystallographic analysis confirmed that Glu463 is located at the equivalent position of Glu506 of CueO [13].

2. Materials and methods

2.1. Enzyme purification

Mutagenesis, expression and purification of the Glu506Gln mutant and purification of the WT enzymes from *E. coli* were carried out as previously described [10]. The WT enzyme of BO from *M. verrucaria* was prepared as previously described [14]. The preparation method for the Glu463Gln mutant will be published elsewhere. The enzyme stock solution was stored at −80 °C until use. For ATR-FTIR measurements, the enzyme solution was concentrated by centrifugal filtration (Vivaspin500, 50 kDa MWCO) of 100 µl of a 1 mM solution in 50 mM potassium phosphate buffer in H_2O at pH 6 or 8, or in D_2O at pD 6. Filtration was repeated several times to exchange the buffer as desired. The pD value was adjusted assuming $pD = pH_{meter\ reading}+0.4$ [15]. Potassium ferrocyanide as a redox mediator was added to the enzyme solution at a final concentration of 1 mM before injection into the ATR cell. The samples were kept at 0–10 °C during preparation.

2.2. ATR-FTIR spectroscopy

ATR-FTIR spectra were recorded with a Bruker VERTEX80 spectrometer equipped with a liquid nitrogen cooled photovoltaic detector. The spectral resolution was 4 cm^{-1} with an accuracy of ±1 cm^{-1}. A horizontal ATR unit with a 3 mm diameter 3-bounce Si internal reflection element and a ZnSe prism (DuraSampleIR, Smiths Detection) was mounted on the sample chamber. The electrochemistry ATR-FTIR apparatus is detailed elsewhere [16,17]. Applied voltage was controlled by a potentiostat (HA150G, Hokuto Denko) with three electrodes (a glassy carbon disk as a working electrode, a piece of platinum foil as a counter electrode and an Ag/AgCl couple as a reference electrode), which were combined in a laboratory made electrochemistry-ATR cell. Working potentials for oxidation and reduction were +550 and −50 mV (vs NHE), respectively. A single reductive/oxidative cycle produced a pair of "reduced *minus* oxidised" and "oxidised *minus* reduced" difference spectra (200 interferograms each). Typically, raw reductive spectra and the inverse of oxidative spectra from 20–30 redox cycles were averaged to improve the S/N ratio. The final "reduced *minus* oxidised" spectra were obtained after subtraction of base line drift due to water vapour and/or buffer if necessary. All measurements were taken at room temperature (25 °C).

3. Results and discussion

Reduced *minus* oxidised difference IR spectra of *E. coli* CueO and *M. verrucaria* BO were measured by electrochemistry-combined ATR-FTIR spectroscopy. To examine the protonation states of the specific carboxy group, the spectra obtained from mutant enzymes of Glu506Gln of CueO and Glu463Gln of BO were compared with WT enzymes. Under aerobic conditions, both the WT and Glu-to-Gln mutants reacted with O_2 and converted it into water molecules as long as electrons were supplied by the ferrocyanide. Ferrocyanide is expected to be an effective electron donor to T1 Cu since the redox potential values of the T1 Cu in CueO and BO are estimated to be around +450 and +650 mV (vs NHE), respectively [18,19]. To generate the reduced form of the enzyme in the ATR cell, the applied potential was set at −50 mV to reduce ferricyanide to ferrocyanide. As the enzyme consumed O_2, the environment inside the cell became anaerobic. The lack of substrate O_2 and a reductive potential left the enzyme in the reduced state. The reductive reaction took approximately 5 min. In the anaerobic condition, ferricyanide alone could not oxidise the reduced form. To re-oxidise the reduced form, air (O_2) was injected into the cell and a potential of +550 mV was applied. The enzyme then returned to the aerobically oxidised form. The obtained "reduced *minus* oxidised" difference spectra are shown in Figs. 2 and 3 (1825–1150 cm^{-1} range) and are summarised in Fig. 4 (1805–1715 cm^{-1} range) to clarify the carboxylic acid C=O stretch region. Band assignment and characterisation of the IR spectra were aided by H/D exchange and the pH change of the buffer media. Since the X-ray crystal structure of BO is not yet available to the public, discussion of

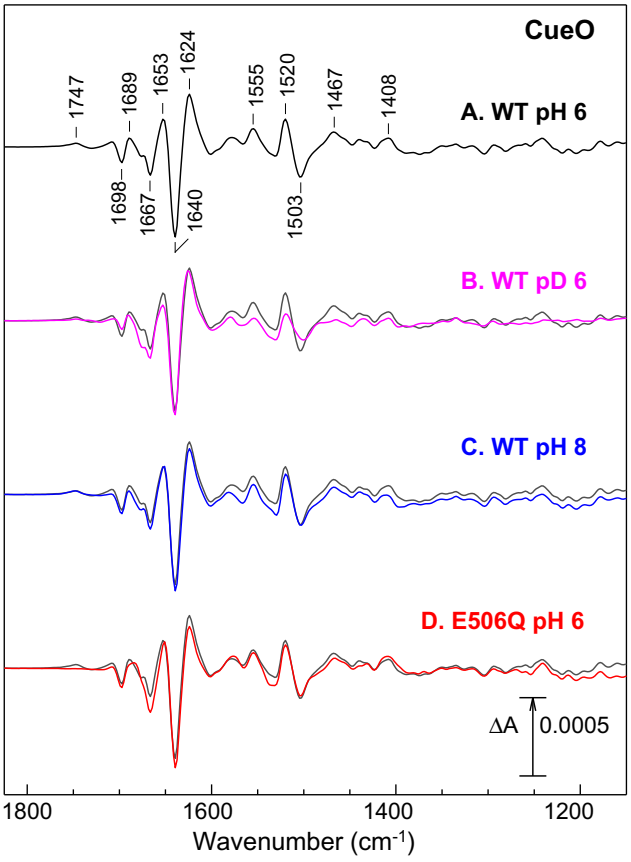

Fig. 2. Electrochemistry-induced "reduced *minus* oxidised" difference FTIR spectra of CueO. ATR-FTIR difference spectra of the wild-type enzyme (A) in 50 mM potassium phosphate buffer at pH 6, (B) at pD 6 in D_2O media and (C) at pH 8 and (D) Glu506Gln mutant enzyme at pH 6. Major peaks and troughs in cm^{-1} are labelled in trace A. For traces B–D, trace A was overlaid in gray for comparison.

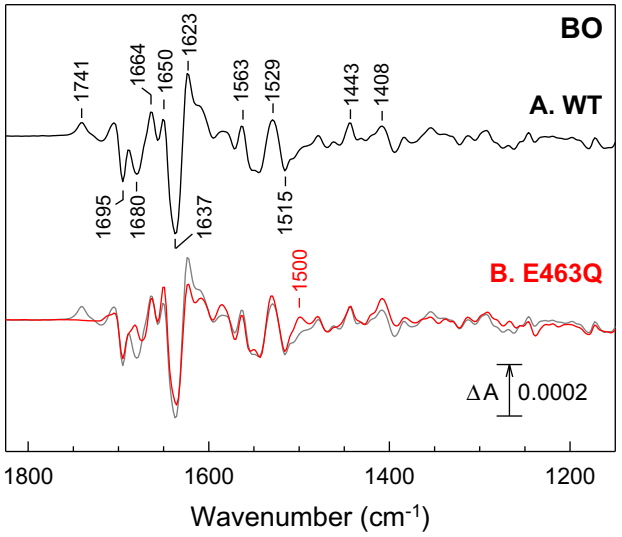

Fig. 3. Electrochemistry-induced "reduced *minus* oxidised" difference FTIR spectra of BO. ATR-FTIR difference spectra of the wild-type (A) and Glu463Gln mutant enzymes (in red, B) in 50 mM potassium phosphate buffer at pH 8. Major peaks and troughs in cm^{-1} are labelled. For trace B, trace A was overlaid in gray for comparison.

the chemical reactions related to the structure of the enzymes will be focused mainly on CueO rather than BO[1].

[1] The X-ray crystal structure of BO has been released as PDB entry 3ABG [13] during the editorial process of this manuscript.

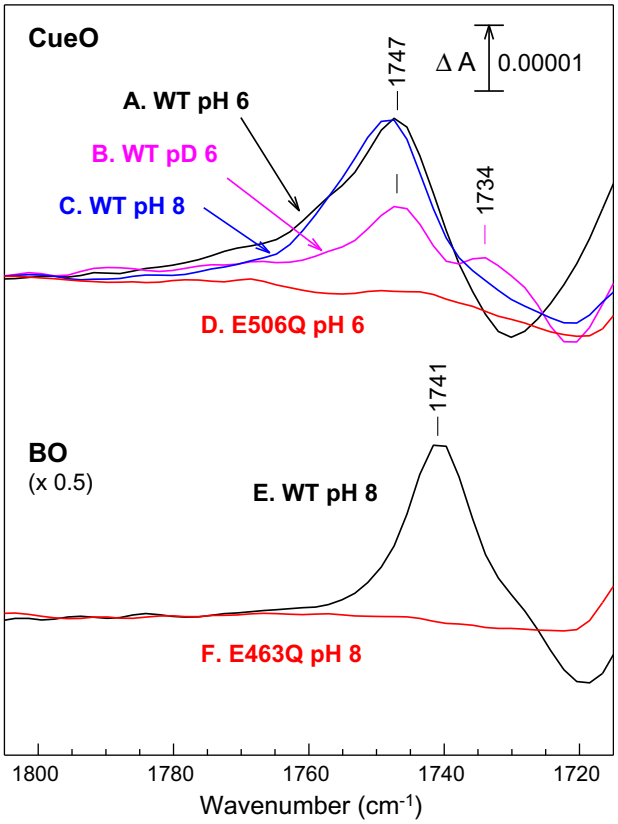

Fig. 4. The protonated carboxylic acid C=O stretch region of "reduced *minus* oxidised" difference FTIR spectra of CueO and BO. The ATR-FTIR difference spectra shown in Fig. 2 (CueO, upper panel) and 3 (BO, lower panel) are rearranged. CueO WT at pH6 (A), at pD 6 (B) and at pH 8 (C) and CueO Glu506Gln at pH 6 (D), BO WT at pH 8 (E) and BO Glu463Gln at pH 8 (F).

3.1. WT CueO

The "reduced *minus* oxidised" difference spectrum of the WT of CueO at pH 6 in H_2O media is shown in Fig. 2, trace A. Major IR features were observed at 1747(+), 1698(−), 1689(+), 1667(−), 1653(+), 1640(−), 1624(+), 1555(+), 1520(+), 1503(−), 1467(+) and 1408(+) cm^{-1}. The spectral features were practically the same as those obtained at pH 8 (trace C). The peaks/trough at 1653(+)/1640(−)/1624(+) cm^{-1} in the amide I region could be assigned to structural changes in the peptide backbone. As the region from 1630 to 1640 cm^{-1} is typical of the β-sheet structure, it is possible that the spectral feature could indicate structural changes in peptide regions containing His ligands of T2/T3 on the β-strands. X-ray crystallographic studies of the oxidised and reduced forms of ascorbate oxidase have shown that the distances between T2/T3 Cu of the reduced form are expanded (0.5–1.4 Å) compared to those of the oxidised form [6]. Such structural change could be expected for other multicopper oxidases. The band at 1520(+)/1503(−) cm^{-1}, whose intensity was reduced in D_2O media (trace B), could be partly due to an amide II change.

In the protonated carboxy C=O stretch region (Fig. 4), a positive band at 1747 cm^{-1} with a shoulder at 1759 cm^{-1} was observed at pH 6 (trace A). In D_2O media (pD 6), bands were observed at 1747 and 1734 cm^{-1}, which were considered to be the bands seen at pH 6 downshifted by H/D exchange (trace B). These results indicate that the acidic residue(s) was unprotonated in the oxidised form and protonated in the reduced form. It is less likely that insufficient H/D exchange caused the residual 1747(+) band because the H/D exchange was estimated to have proceeded to greater than 90%

completion as estimated by the downshift of the amide II band in the absolute IR spectra (data not shown). At pH 8, the 1747(+) band was basically retained with a slight modification (trace C). This might indicate that a hydrogen bond network around the protonated carboxy group was affected by the pH perturbation. The pKa values of the carboxy group in the oxidised and reduced forms were estimated to be <6 and >8, respectively.

3.2. Glu506Gln mutant CueO

The Glu506Gln mutation resulted in only minor modifications of the "reduced *minus* oxidised" difference spectrum compared to that of WT, except for the entire loss of the 1747(+)/1759(sh) feature (Figs. 2 and 4, trace D). No response in the carboxy C=O stretch region was confirmed in D_2O media at pD 6 and in H_2O media at pH 8 (data not shown). These results strongly suggest that the carboxy group of Glu506 is responsible for the IR feature. In the WT, if the proton is uptaken from external media to Glu506, the next residue, Asp507, would be expected to mediate the proton transfer to Glu506 (see Fig. 1). The protonation state changes in Asp507 may only be transient, and the protonation state of Asp507 is likely to be unchanged in the reduced and oxidised forms. In the Glu506Gln it may be possible that the amide head group of Gln506 retains a part of the hydrogen bond network that is arranged by Glu506 in the WT. Thus the structural rigidity of the Gln506 scaffold would not allow proton transfer as a water (proton) channel would be blocked.

The relatively broad 1747(+)/1759(sh) feature in the WT spectrum indicates that the band contains multiple components and/or heterogeneity of the C=O stretch. This result could arise from the carboxy group of Glu506 with a multiple and bifurcated hydrogen bond arrangement (see Fig. 1). A slight modification of the IR feature at pH 8 compared to that at pH 6 might indicate the perturbation of protonation states for the hydrogen bond donor/acceptor of Glu506.

3.3. WT and Glu463Gln mutant BO

The "reduced *minus* oxidised" difference spectrum of the WT BO at pH 8 in H_2O media is shown in Fig. 3, trace A. Major IR features were observed at 1741(+), 1695(−), 1680(−), 1664(+), 1650(+), 1637(−), 1623(+), 1563(+), 1529(+), 1515(−), 1443(+) and 1408(+) cm^{-1}. The features of the peaks/trough at 1650(+)/1637(−)/1623(+) cm^{-1} in the amide I and at 1529(+)/1515(−) cm^{-1} in the amide II regions are similar to those in CueO. This similarity suggests that the major structural changes of the peptide backbone in BO and CueO are similar, as expected from the homologous amino acid residues and molecular structure around the Cu sites in the two enzymes [3,4,13]. In the spectrum of the Glu463Gln mutant, a slight increase of the absorption bands at 1500 cm^{-1} compared to that of WT may indicate a variation in the structural changes caused by the mutation (trace B).

In the carboxy C=O stretch region (Fig. 4), the 1741(+) cm^{-1} band of the WT (trace E), which could be assigned to the protonated carboxy group that is expected to have a pKa value higher than 8, was lost by the Glu463Gln mutation (trace F) as observed in the CueO enzymes. The 1741(+) band was narrower and at a lower wave number relative to the 1747(+) band of CueO. These differences might indicate that the carboxy group of Glu463 of the reduced form of BO could be in a relatively simple hydrogen bond network with a stronger interaction compared to the case of CueO. Overall, the results support the proposition that the Glu463 of BO functions in a similar manner as Glu506 of CueO. It is also confirmed that no acidic residues other than the Glu are involved in the protonation state change upon reduction.

We expected to detect the COO^- stretch bands of the Glu carboxylate in the 1550–1400 cm^{-1} region of the oxidised form of CueO and BO enzymes by examining the double difference between the spectra of WT and Glu mutant. However, we were unable to identify the bands because their intensity might have been low compared to those of other vibrational modes.

3.4. Proton transfer in multicopper oxidases

Glu506 of CueO and Glu463 of BO in the oxidised forms are expected to be unprotonated with a pKa presumably similar to that of glutamic acid in aqueous solution, i.e. at around 4. In the reduced form, the pKa value of Glu was elevated to higher than 8. This increase suggests that the protonation states of Glu506 of CueO and Glu463 of BO are strongly coupled with the redox electronic state of the T2/T3 Cu ions. The uptaken proton on the Glu would then be ready to be transferred to the activated oxygen species at the T2/T3 Cu site during O_2 reduction, which is consistent with the reaction model previously proposed by Kataoka et al. [10]. The present study suggests that there are no protonation state changes in any other acidic residues in both CueO and BO, thus Glu506 of CueO and Glu463 of BO might be able to translocate more than one proton from the external media to the T2/T3 site. The results also suggest that the function of Asp112 in CueO, which is adjacent to T3 Cu (see Fig. 1), could be mainly in O_2 binding rather than proton translocation [10,20]. It is not clear, however, how many protons are transferred in each reaction step, and the present results cannot exclude the possibility of participation of other proton pathways.

3.5. Redox changes of T1 Cu

A detailed examination of the redox changes of T1 Cu is not included in this paper. It is noted, however, that the present FTIR spectra do contain contributions from conformational changes due to redox conversion of T1 Cu as well as T2/T3 Cu, although we could not separate the IR changes of T1 Cu from those of T2/T3 Cu. The reduced *minus* oxidised difference spectrum of plant plastocyanin, which contains one T1 Cu as a redox centre [21,22], showed major IR features at around 1700(+), 1660(−) and 1630(+) cm^{-1} in the amide I region [17,23]. The signal intensity of the 1660(−)/1630(+) feature calculated from published data [17] is reasonably comparable (1/3–1/2) to the intensity of amide I change in the CueO/BO data.

In conclusion, electrochemistry-induced ATR-FTIR spectroscopy combined with mutagenesis revealed that Glu506 of CueO (Glu463 of BO) was protonated in the reduced form and unprotonated in the oxidised form of the enzyme. The results support the hypothesis that the acidic residue in the position participates in proton translocation to the activated oxygen species during the O_2 reduction cycle of multicopper oxidases.

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